346. *Physicochemical Aspects of Bacterial Growth. Part IV. Conditions determining Stationary Populations and Growth Rates of* Bact. Lactis Aerogenes *in Synthetic Media.*

By R. M. Lodge and C. N. Hinshelwood.

The maximum population (n_s) of *Bact. lactis aerogenes* which an artificial medium containing lactose and ammonium tartrate will support at first increases in proportion to and then becomes independent of the concentrations of these constituents: the rate of growth varies much less than n_s.

The curves showing the relation between n_s and the initial p_H of the medium have characteristic shapes for three media containing (a) lactose–ammonium tartrate, (b) concentrated glucose–phosphate, and (c) dilute glucose–phosphate, (c) showing a considerable range, absent in (b), where n_s is independent of p_H. The growth rate is independent of n_s over a wide range.

These and other observations show that the onset of the stationary phase is due principally to (1) exhaustion of substances necessary for growth, (2) accumulation of toxic products, and (3) adverse p_H, any of which according to circumstances may become the limiting factor. Since, under adverse conditions, maximum growth is independent of the inoculum size within the considerable range studied, it is inferred that progressive deterioration of the cells from one generation to another does not contribute to the stopping of the growth.

From the fact that at adverse p_H the stationary population is much reduced without any corresponding change in the mean generation time, it is inferred that different stages of the cell division process may have separate locations, one being shielded from the influence of the medium p_H.

WHEN *Bact. lactis aerogenes* is grown in a medium containing glucose, potassium phosphate, ammonium sulphate, and magnesium sulphate, the stationary population of organisms is found to increase linearly with the amount of glucose or phosphate over a considerable range, and beyond a certain point to become independent of concentration. This shows that in more dilute solutions the exhaustion of the medium is the factor responsible for the cessation of growth and the onset of the stationary phase, but that at higher concentrations other factors may take control (Part I, J., 1938, 1930).

It is of interest that over ranges of medium concentration where the stationary population changes by more than a hundredfold, the rate of growth, as measured by the mean generation time, hardly changes.

To throw further light on the factors determining the stationary population and on the relation between stationary population and growth rate, the work on *Bact. lactis aerogenes* has been extended by the following series of observations: (1) The effect of varying the concentrations of the constituents in a lactose–ammonium tartrate medium. (2) The influence of the hydrion concentration (*a*) in the glucose–phosphate medium (Gladstone, Fildes, and Richardson, *Brit. J. Exp. Path.*, 1935, **16**, 335), (*b*) in the lactose–tartrate medium (Winslow, Walker, and Sutermeister, *J. Bact.*, 1932, **24**, 185). (3) The influence of inoculum size, temperature, and nature of carbohydrate.

These determinations, together with a few auxiliary experiments, lead to the following general conclusions:

(*a*) Exhaustion of constituents necessary for growth, change of p_H, and accumulation of toxic products are factors whose interplay determines the onset of the stationary phase, and one or other of which may become the limiting one according to circumstances.

(*b*) The lack of parallelism between variations of growth rate and of stationary population with change of p_H suggests a separate localisation of different stages essential to the growth mechanism.

Stationary Population and Concentration.—The culture was the same strain of *Bact. lactis aerogenes* as that used in Part I (*loc. cit.*), and had originally come from the national collection of type cultures. It was checked against a second supply and showed no difference in spite of the repeated sub-culturings which it had undergone.

The lactose–tartrate medium (Winslow, Walker, and Sutermeister, *loc. cit.*) was made from the following solutions: (*a*) lactose, 20 g./l.; (*b*) ammonium tartrate, 25 g./l.; ammonium dihydrogen phosphate, 4·0 g./l.

The methods of sterilising and technique generally were as described previously. The inoculum used was a stock bouillon culture, sub-cultured for 24 hours in the artificial medium. Experiments were made at 40·0°.

The results of varying the concentrations of lactose and of tartrate are given in Tables I, II, and III. The counts recorded are the actual numbers per unit field; multiplied by $1{\cdot}25 \times 10^6$, they give the number of organisms per c.c. Fig. 1 shows that the stationary population increases linearly at first and then much more slowly. The mean generation

times tend to increase as the concentration falls, but nothing like so rapidly as the stationary population.

TABLE I.

Medium containing ammonium tartrate, 4 g./l., and ammonium dihydrogen phosphate, 0·016 g./l.; $T = 40·0°$.

Lactose, g./l.	1	2	4	6	8
Stationary population	15·7	31·6	53·6	78	80
Mean generation time, mins.	—	—	227	173	126

TABLE II.

Medium containing lactose, 4 g./l., and ammonium dihydrogen phosphate, 0·016 g./l.; $T = 40·0°$.

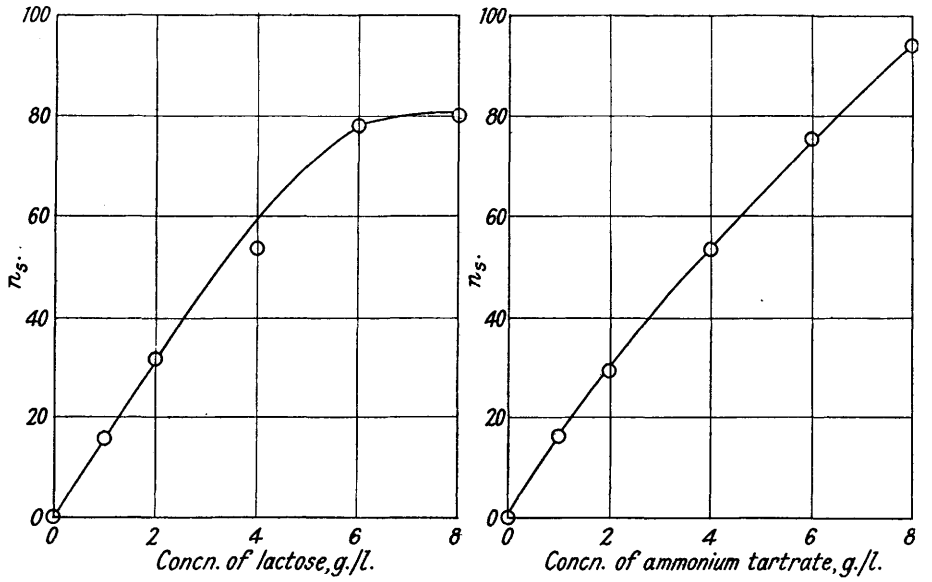
Ammonium tartrate, g./l.	1	2	4	6	8
Stationary population	16·2	29·2	53·6	75·5	94
Mean generation time, mins.	—	—	227	239	120
No. of determinations	6	2	2	2	1

TABLE III.

Medium containing lactose, 4 g./l., and ammonium tartrate, 4 g./l.; $T = 40·0°$.

$NH_4H_2PO_4$, g./l.	0·004	0·016	0·032
Stationary population	68	53·6	42
Mean generation time, mins.	487	227	276
No. of determinations	2	2	2

FIG. 1.



Influence of concentration of lactose and of ammonium tartrate on stationary population.

Influence of p_H *on Stationary Population and Growth Rate.*—For the determination of the influence of hydrogen-ion concentration the media were made up as follows: (*a*) Glucose, 24·6; ammonium sulphate, 0·985; potassium dihydrogen phosphate, 4·43; magnesium sulphate, 0·059 g./l. (*b*) Lactose, 8·0; ammonium tartrate, 10·0; ammonium dihydrogen phosphate, 0·016 g./l., and a similar medium to (*a*) but with glucose, 0·5 g./l.

The p_H was adjusted by adding N/10-hydrochloric acid or sodium hydroxide, blank experiments having shown that sodium and chlorine ions at the concentrations thereby

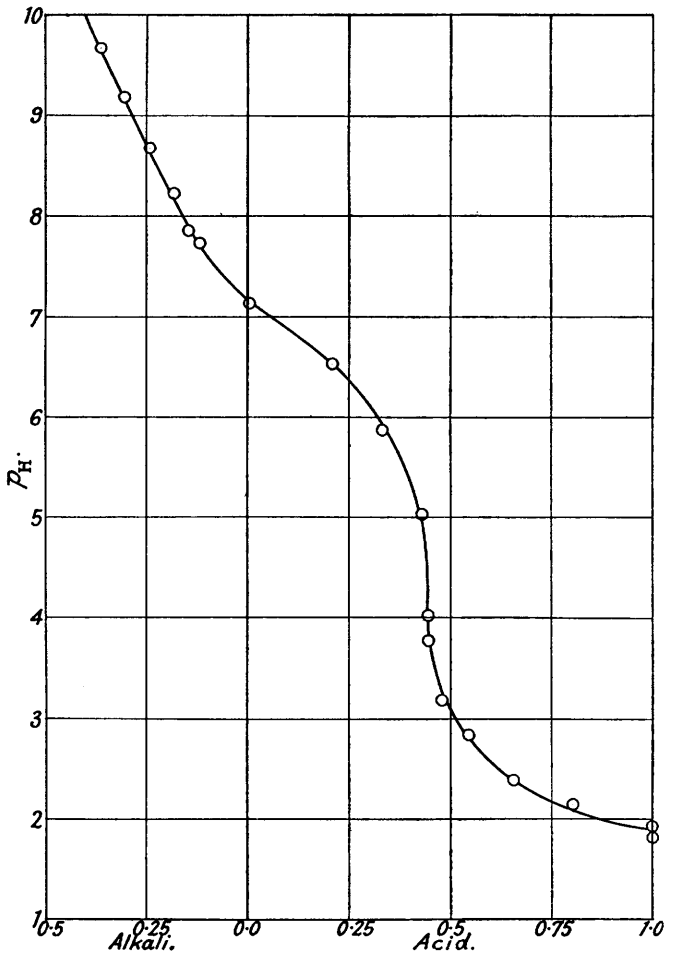
introduced had no effect on growth. Figs. 2A and 2B show the titration curves of the media and give an idea of the efficiency of buffering in various regions, a matter which may be relevant in assessing the results.

The p_H was determined by means of a glass electrode used in conjunction with a valve electrometer giving direct readings in p_H units. The inocula were prepared in the appropriate artificial medium near neutrality.

The determinations were made at 40·0°, and a slow current of sterile air from outside the laboratory was passed through the media during growth.

FIG. 2A.

Titration curve of glucose–phosphate medium.



Volume of 0·1N-*alkali or acid in a total of* 20·3 *c.c. of glucose–phosphate medium.*

The results are in Tables IVa, IVb, and V. In the first place Figs. 3 and 4 show that the relation between p_H and stationary population is rather different for the two media

TABLE IVa.

The relation between the initial p_H and the stationary population in the glucose–phosphate medium; $T = 40°$.

Initial p_H	9·18	8·67	8·21	7·50	7·44	7·11	6·55	6·53	5·92	5·86
Stationary population	10·2	19·0	23·2	1440	1280	1000	495	479	225	210
Initial p_H	5·48	5·28	5·07	4·02	3·79	3·16	2·82	2·42	2·13	
Stationary population	140	112	79	22	13·4	7·9	4·6	3·5	2·7	

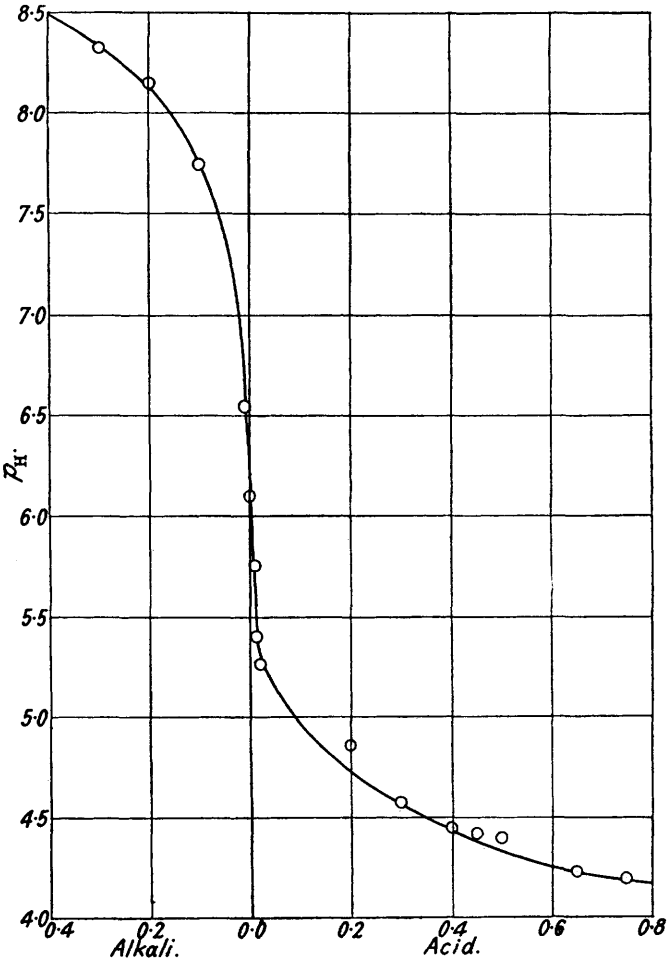
TABLE IVb.

The relation between the initial p_H and the stationary population in the dilute glucose medium; $T = 40°$

Initial p_H	3·10	4·90	5·48	5·53	5·56	5·70	5·83	6·14
Stationary population	4	31	53	63	66	64	108	121
Initial p_H	6·40	6·81	7·12	7·41	7·58	8·27	9·25	9·55
Stationary population	135	131	135	124	130	100	7	4

FIG. 2B.

Titration curve of lactose–tartrate medium.



Volume in c.c. of 0·1N-*alkali or acid in a total of* 37·5 *c.c. of lactose–tartrate medium.*

TABLE V.

The relation between the initial p_H and the stationary population in the lactose tartrate medium; $T = 40°$.

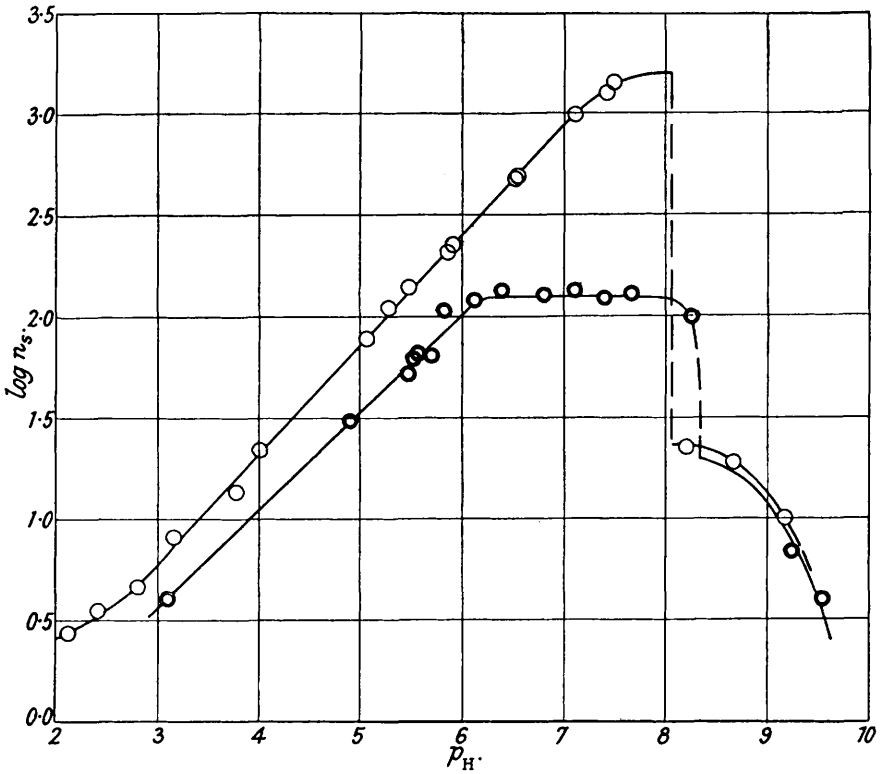
Initial p_H	4·19	4·23	4·41	4·43	4·44	4·45	4·56	4·86	4·92	5·02
Stationary population	10·0	5·0	120	119	112	108	140	102	94	99
Initial p_H	5·17	5·41	5·74	6·00	6·12	6·54	7·74	8·24	8·32	8·75
Stationary population	86	117	63	95	80	67	50	5·8	9·5	6·2

and for the two glucose concentrations, but in all cases the fall on the alkaline side is abrupt. Part of the curve is unobtainable, namely, that corresponding to the region just

on the alkaline side of neutrality where the acid produced by a moderate growth of organisms is enough to shift the p_H progressively towards the optimum.

Once again, there is a remarkable lack of correspondence between the abundance of growth, as measured by the stationary population, and the actual rate of growth (mean generation time). Even the feeble growth in the regions of more adverse p_H is attained as quickly as a corresponding growth at the optimum p_H. This is illustrated in Table VI: the mean generation times show a random variation only.

FIG. 3.



Influence of p_H *on stationary population in two glucose–phosphate media.*

TABLE VI.

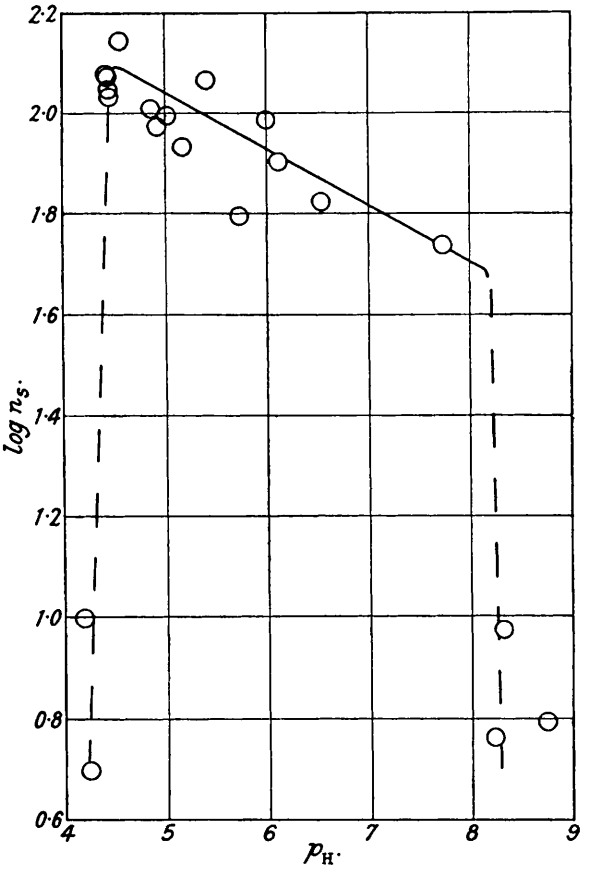
The variation in the mean generation time with the initial p_H of the glucose–phosphate medium; $T = 40°$.

Initial p_H	9·18	7·50	7·44	7·11	6·53	5·86	5·07	4·02
Mean generation time, mins.	56	40	67	·54	38	34	58	41

With the lactose–tartrate medium the actual mean generation times were difficult to determine in the adverse p_H region because of the smallness of the total growth, but the times required to reach a given count certainly did not increase as the conditions became more adverse. For example, at p_H 8·3 a culture which never grew beyond a count of 10, had reached a count of 9·5 in 880 minutes, whereas at p_H 6·45 a culture which ultimately grew to a count of 152 reached the count 9·5 in 1000 minutes. In the first experiments the growth rates themselves were less reproducible than had been hoped in view of the care taken in standardising the inoculum. The difficulties largely disappeared when a nichrome loop was replaced by a platinum one, doubly distilled water was used instead of the laboratory supply, a greased tap on a measuring device was eliminated, and culture tubes were degreased repeatedly to remove the minute film which sometimes comes from inserting a cotton-wool plug into a hot tube after sterilisation.

Experiments on the Cause of the Onset of the Stationary Phase.—To determine whether growth could be said to stop when the p_H of the medium was brought to a definite value by the acid which the organism itself produces, measurements were made of the initial p_H and of the p_H immediately after the end of the logarithmic growth phase (cf. Wolf and

FIG. 4.



Influence of p_H *on stationary population in lactose–tartrate medium.*

Harris, *Biochem. J.*, 1917, **11**, 213). The results are given in Table VII and show that there is no such definite value. A culture can, in fact, grow on a fresh medium at a p_H

TABLE VII.

Medium								
Glucose–phosphate	Initial p_H	4·02	5·86	6·53	7·11			
	p_H at onset of stationary phase, at 40°	3·88	4·21	5·54	6·26			
Lactose–tartrate	Initial p_H	4·86	4·92	5·17	5·40	5·74	6·00	6·12	6·54
	p_H at onset of stationary phase, at 40°	4·84	4·15	5·40	5·51	5·64	5·50	5·70	5·56

considerably more on the acid side than many of the " final " p_H values recorded in the table. This shows that the p_H is by no means the sole limiting factor, as has already been proved by the experiments on the influence of the concentration of the medium constituents.

If exhaustion of a necessary constituent of the medium is thought of as the sole factor limiting bacterial growth, changes of p_H could easily be understood to alter the rate of growth by shifting equilibria which determine the availability of this constituent. But since such an equilibrium shift would not alter the total supply, it is difficult to see why

the same stationary population should not ultimately be reached. In fact, it is the stationary population and not the growth rate which varies. This raises the question of the reasons for the onset of the stationary phase.

One way in which an adverse environment could bring about an earlier cessation of growth would be by causing a progressive degeneration of the organisms in such a way that the nth generation becomes incapable of further division. In these circumstances the stationary population would be equal to the initial count $\times 2^n$; *i.e.*, it would be proportional to the size of the inoculum. Experiments were made which showed definitely that no such relation exists. From Table VIII it may be seen that a given solution will

TABLE VIII.

Influence of inoculum size.

Initial p_H.	Initial count.	Final count.	Increment.
	Glucose–phosphate medium, at 40°.		
6·55	1	492	491
	20	500	480
5·48	1	140	139
	17	140	123
	Bouillon, at 45·5°.		
	8	59	51
	52	100	48

allow a definite increment of population irrespective of the inoculum size over the wide range studied.

Progressive changes from generation to generation being thus eliminated, the stationary phase must be determined either by the exhaustion of something needed for growth, or by the accumulation of some deleterious substances. Under optimum conditions of p_H and at not too high foodstuff concentrations, exhaustion is frequently the limiting factor, as already shown. Under adverse p_H conditions it cannot be, since the p_H is unlikely to affect the total supply of any substance. The only way in which it could do this would be by facilitating useless side reactions in which the constituents of the medium were wasted. That such waste is not in fact responsible is shown by an experiment in which a culture was grown in a glucose–phosphate medium of p_H 5·28 reaching a count of 112. The culture was then brought to p_H 6·81, whereupon growth proceeded to a new stationary count of 860. Therefore, before neutralisation, although growth had stopped, the essential growth constituents could have been neither used up nor wasted. The way in which exhaustion ceases to be a limiting factor when the p_H is adverse is shown in Table IX.

TABLE IX.

Glucose–phosphate medium, at 40·0°.

	Stationary count.	
	Glucose, 24·6 g./l.	Glucose, 0·985 g./l.
p_H 7·11	1000	172
p_H 5·20	95	95

The converse case is illustrated in Fig. 3. For the high glucose concentrations the stationary count varies with p_H over the whole range; but for the dilute glucose solutions the horizontal plateau in the curve shows that the p_H has ceased to be a limiting factor in this range and that the growth ceases through exhaustion, and independently of the p_H.

In considering the accumulation of growth-inhibiting substances, it is convenient to discuss acid and other toxic substances separately, since the p_H determines the whole behaviour of the amphoteric proteins in the cell.

Stationary Population and Growth Rate.—It must be emphasised that there are two problems presented by these results which have some considerable significance in the analysis of growth mechanisms.

First, we have the growth rate independent of p_H over a range in which the stationary population varies very widely. Secondly, the p_H influences the total number of organisms

which the medium can support although it can hardly be supposed to do other than shift equilibria, which one might have expected to determine rate of growth rather than total numbers.

In a growth curve the stationary phase sets in by rapid drop of the growth rate from a constant value to nearly zero. The above results mean that an adverse p_H, while not changing the steady rate, causes the rapid drop to occur much earlier.

It is known that the relation between growth rate and the concentration of substances necessary for growth is a curve which rises rapidly from zero to become almost constant at quite low concentrations (Penfold and Norris, *J. Hyg.*, 1912, **12**, 527), so that over very wide ranges the rate of growth may be independent of the concentrations, but below a critical value of the latter drops rather rapidly.

The most important direct influence of p_H is likely to be on amphoteric protein structures, whose reactivity in various respects will depend upon their state of ionisation; p_H might be expected to influence growth through such a mechanism, but if it did, we should expect a steady variation in actual growth rate over the whole p_H range, and this is just what we did not find.

We are thus led to the idea of an intermediate substance the rate of whose synthesis depends upon the p_H, and which, being labile, attains in the cell to some transient equilibrium concentration depending upon the p_H and other factors. The bacterial growth rate is, according to the general rule, independent of this concentration over a wide range. The stage of growth, however, at which this concentration drops below the critical value sets in progressively earlier the more adverse the conditions governing its primary synthesis.

These points can be made clear by a rough calculation. Let the intermediate substances be synthesised at a rate which is a function, f(c), of the foodstuff concentration, F(p_H) of the p_H, and of the concentration, p, of toxic products, which can be approximately represented by a linear expression.

This gives for the rate of formation, $K \cdot f(c) \cdot F(p_H) \cdot (1 - ap)$, where K and a are constants. Owing to the characteristic form of the curves connecting growth rate and food concentration, the former will be independent of this until it drops below a critical value. This it does when the product of all three factors becomes small enough. In extreme cases this may be when f(c) approaches zero, *i.e.*, exhaustion, when F(p_H) becomes small enough, or when ap approaches unity. When F(p_H) is small, *i.e.*, adverse conditions, it requires a smaller accumulation of toxic products to reduce the product to the critical value.

When exhaustion is the limiting factor the stationary population should become less dependent upon p_H, as is indeed found. With dilute media there should be a considerable horizontal plateau in the p_H–stationary population curve, as is found with the more dilute glucose–phosphate medium and towards which there is a tendency with the lactose–tartrate medium (Figs. 3 and 4).

With more concentrated media, the accumulation of metabolic products and the change in p_H brought about by the acid-forming reactions of the cells begin to play a more important part. Acid and other products both exert an effect. Neutralisation, as already stated, leads to further growth. This shows that the acid was in these circumstances partly responsible for the onset of the stationary phase. The following experiment indicates that other toxic products are also concerned. A culture was grown to its stationary phase (count about 1000) in a medium containing 24·6 g./l. of glucose. The solution was filtered through a porcelain candle and the filtrate divided into two parts. One was reinoculated and showed no further growth at all: the other was first boiled for 10 minutes, and then reinoculated, whereupon further growth occurred to a count of 109. The p_H, 5·5, was not changed by boiling, and this further growth, which has been recorded by others, corresponds approximately to what occurred in a fresh medium of this p_H.

These experiments as a whole thus give us a fairly complete picture of how the exhaustion of medium constituents, the change of p_H of the medium, and the accumulation of toxic metabolic products interplay in determining the stationary population (Tables IVa, IVb, and V), one or other factor becoming the limiting one according to the conditions.

The relative constancy of the growth rate under conditions which cause wide changes in the stationary population leads to the idea that the p_H does not change directly the reactiveness of the centres where the intermediate substance actually promotes cell division. These centres appear to be at a point sheltered from, or unresponsive to, the direct action of the p_H. The growth process thus seems to be resolved into separately localised stages.

It is interesting to note the contrast between this unresponsiveness, which taken by itself might have suggested a definite mean generation time characteristic of the normal organism itself, and the wide changes in growth rate caused by differences in the chemical nature of the medium. Thus, in bouillon at 40·0°, *Bact. lactis aerogenes* has a mean generation time of approximately 20 mins., in the glucose–phosphate medium of about 50 mins., and the lactose–tartrate of about 200 mins. The following figures show the influence of change of carbohydrate in a standard phosphate–ammonium sulphate medium :

Carbohydrate	Glucose	Galactose	Lactose
Mean generation time (mins.)	39, 45, 41	45	76, 67

The behaviour with the two monosaccharides is similar, but growth with the disaccharide is considerably slower. Generally speaking, with change in the chemical nature of the medium the stationary population varies in a way which runs parallel with the change in growth rate. The same observation applies to the toxic action of alcohols, where the stationary population and the growth rate fall off in a more or less parallel manner.

Thus we have two contrasting types of behaviour, one where the stationary population and the growth rate show parallel changes and one where they are independent.

The question arises now as to which type is shown when the temperature is varied. It is known that the growth rate increases with temperature at first according to the usual exponential law for chemical reaction velocity, and then passes through a maximum and finally declines suddenly (Barber, *J. Infect. Dis.*, 1908, **5**, 379). Experiments were now made to determine the variation of the stationary population. The medium used was veal bouillon. The results are given in Table X. They show that, taken over

TABLE X.

The effect of temperature on bacterial growth.

T	15·0°	20·0°	25·0°	30·0°	35·0°	40·0°	45·0°	45·5°	46·0°	47·5°
Mean generation time, mins.	300	130	—	47	45·7	25·8	42	—	—	—
Stationary population	690	803	1045	1010	810	658	132	59	0	0

the whole range, the rate and the total count vary in a generally similar way, but that the optima occur at different places, and that in certain regions the two are changing in opposite directions.

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